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Electric shock-mediated transfection of cells

Characterization and optimization of electrical parameters

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The effect of various parameters on the electric shock-mediated permeabilization and transfection of CHO cells has been investigated. Up to 70% of the cells can be maintained transiently permeable to erythrosin B for periods of at least 1 h at 20 °C. Electrical conditions optimal for transient permeabilization were also optimal for efficient DNA transfection by pSV2*neo*. However, the DNA must be present during exposure to the electric field for efficient transformation. The same requirement existed for voltage-induced DNA toxicity. The results suggest that DNA moves into the cells by electrophoresis, not by simple diffusion. Based on these observations a simple, rapid procedure for optimizing the conditions for electric shock-mediated DNA transfer into cells has been developed.

INTRODUCTION

The transfer of new genetic material, as well as non-permeant biochemical inhibitors or foreign proteins, into eukaryotic cells has become an important experimental procedure. As such, a wide variety of techniques have been developed to permit the uptake of exogenous molecules. The exposure of cells to large pulses of electrical energy has been suggested by a number of studies [1–10] to permit uptake of molecules by a broader range of cell types than other techniques. It is generally believed that the electric field generates pores in the plasma membrane, allowing diffusion of extracellular molecules into the cytoplasm [1,3,7] and that the lower dependence on cell type is due to the physical nature of the procedure [9].

Equipment used to generate the electrical field, whether of commercial or experimental design, normally provides facilities allowing control over the amplitude, duration and number of pulses as well as the time between pulses. Optimization of these parameters, as well as other variables such as buffer conductivity etc., is required for each cell type before efficient permeabilization can be obtained ([9]; our unpublished observations). We show here that evaluation of the transient ability of cells to stain with erythrosin B, or of toxicity due to electroporation in the presence of high concentrations of DNA, provide rapid and reliable methods of optimizing the electrical parameters for permeabilization and gene transfer respectively. Simple procedures for such optimization are described. In addition we present evidence that the effect of the electrical field on gene transfer is not just to permeabilize the acceptor cell, but also to induce uptake of the DNA by virtue of its electrophoretic mobility.

MATERIALS AND METHODS

Cell culture

CHO K1 (a Chinese hamster ovary cell line [11]), α modification of Eagle's medium, heat-inactivated newborn bovine serum, Earle's balanced salt solution without Ca^{2+} and Mg^{2+} and trypsin solution were obtained from Flow Laboratories. Geneticin sulphate (G418) was obtained from GIBCO as lots varying from 48.3 to 51.7% active drug; stock neutralized solutions of G418 were prepared at 40 mg of active drug/ml in distilled water and stored at −20 °C after sterilization by filtering through a 0.2 μm Acrodisc (Gelman Sciences, Brackmills, Northampton, U.K.). Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air in 60 mm (no. 3002) or 90 mm (no. 3003) tissue culture dishes (Falcon, Becton Dickinson) containing 4 or 8 ml respectively of 'complete medium' [αMEM with 10% (v/v) added newborn bovine serum, without antibiotics]. In some experiments, the CHO cells were cultured in suspension in stirred culture vessels (Techne MCS stirrer) in the same medium.

DNA preparation

Supercoiled plasmid pSV2*neo*, containing a dominant selectable aminoglycoside phosphotransferase gene [12], was prepared by conventional means and analysed for purity by agarose gel electrophoresis and u.v. spectrophotometry of aliquots. Sterile stock solutions of 100 μg of pSV2*neo*/ml were prepared by ethanol precipitation [0.7% (w/v) potassium acetate in 70% (v/v) ethanol] and washing in 70% (v/v) ethanol before air drying in a laminar flow hood and redissolving in electroporation buffer (see below); stock solutions of plasmid were stored at 4 °C. Unless otherwise noted, transfection was carried out with supercoiled plasmid. In some cases plasmid was linearized by restriction with *Bam*H1 (Pharmacia) following the manufacturers recommendations, or converted to the relaxed form using a crude preparation of topoisomerase I, as described [13]. Following restriction, the samples were extracted with phenol and chloroform and precipitated and sterilized with ethanol as described above, except that three washes with 70% (v/v) ethanol were performed to ensure removal of traces of phenol or chloroform. Salmon testes

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DNA (D1626, Sigma) was sterilized by ethanol precipitation and redissolved at the required concentration after thorough drying in a laminar flow hood.

Standard electroporation conditions

Cells were normally harvested from dense cultures towards the end of exponential growth, by washing the cell sheet with 0.02% EDTA in Ca^{2+}- and Mg^{2+}-free Earle's balanced salt solution and incubating for 5 min at 37 °C in fresh EDTA solution. Trypsin was added to a final concentration of 0.05% (w/v) and the dishes were incubated for a further 5 min at 37 °C. The cells were suspended in complete medium, to block trypsin activity, centrifuged at 300 *g* for 5 min, washed in electroporation buffer, centrifuged and resuspended at the appropriate density in electroporation buffer. Electroporation buffer was prepared by adjusting a solution containing 280 mM-mannitol (AnalaR, BDH) and 20 mM-Hepes to pH 7.2 with KOH. The stock cell suspension could be maintained on ice for up to 2 h without any detectable change in viability or ability to reseal after electroporation.

In preliminary studies, it was established that using potassium rather than sodium as the only cation in the electroporation buffer resulted in significant improvements in performance as judged by the following criteria: percentage of transiently permeabilized cells at the optimum voltage, the ability of cells to survive field strengths above the optimum voltage, and in the ability of cells stored for extended periods of time in electroporation buffer to recover from the electric shocks.

Samples (0.1–0.5 ml) containing 10^7 cells/ml and 10 μg of plasmid/ml (this concentration was varied in some experiments) were subjected to electroporation with a commercially available device (TA750 electrotransfection apparatus; KRÜSS GmbH, Hamburg, Germany). The apparatus (used with a transfection chamber comprised of two concentric stainless steel electrodes separated by a gap of 0.5 mm) was capable of generating a series of square wave pulses. The amplitude, duration and number of pulses and the time between pulses are all adjustable by the operator; the settings used in specific experiments are described in the text. Following electroporation, the cell suspension was gently expelled from the chamber into a sterile disposable bijoux bottle (Sterilin) and maintained at the appropriate temperature for the required period before allowing the cells to reseal by diluting 5-fold in complete medium and incubating at 37 °C for 1 h. If conical tubes were used during the post-shock incubation of permeabilized cells a marked drop in viability was observed. This appeared to be due to sedimentation of the cells into a concentrated pellet. Decreased viability was observed in bijoux bottles as well if cell densities were above 5×10^7 cells/ml.

Assay for membrane integrity

Cell membrane permeability was operationally defined as the ability to take up erythrosin B (M_r 880). We found that erythrosin B-stained cells were easier to detect than cells stained by other dyes such as Trypan Blue. Aliquots of cells suspended in electroporation buffer were diluted 5-fold with complete medium and mixed with an equal volume of 0.4% (w/v) erythrosin B (Aldrich Chemical Co.) in phosphate-buffered saline without Ca^{2+} and Mg^{2+}. After 4 min (and before about 10 min) the percentage of stained cells was determined in a haemocytometer under brightfield illumination. For each assay, four measurements of about 100 cells were made. Results are presented as the mean of these values, the standard deviation of the measurements was generally 4% and always less than 10%.

Transiently permeabilized cells were defined as those cells made permeable by exposure to the electric shock(s) that were able to reseal within 1 h at 37 °C in complete medium. Under these conditions, resealing of permeabilized cells (as judged by erythrosin B exclusion) proceeds rapidly with a $t_{\frac{1}{2}}$ of less than 5 min. The residual percentage of stained cells at 30–60 min correlates well with the percentage of dead cells as judged by plating efficiency. The correlation coefficient of the relationship between unstained cells (after the 1 h period for resealing at 37 °C in complete medium) and viable cells (as determined by plating efficiency) in data pooled from six separate experiments was 0.94. For clarity, we have therefore referred to staining results obtained after allowing cells to reseal in terms of 'viability'.

Assay for transformation frequency

After dilution of the electroporated cells in complete medium, aliquots of 50 or 100 μl (containing 1×10^5 or 2×10^5 cells) were plated into 60 or 90 mm dishes respectively; 2 days were allowed for expression of the *neo* gene before replacing the medium with medium containing geneticin (400 μg of active drug/ml). The medium was replaced with fresh medium containing geneticin at the same concentration after 7 days had elapsed from the date of electroporation. The number of colonies of transformed cells was determined 5 days after this by staining the fixed dishes in 1% (w/v) Crystal Violet. Each point represents the number of colonies in a single dish. Repeated transformations under identical conditions varied with an S.D. of $\pm 14\%$ of the mean. Results were expressed as a percentage of the cells exposed to the electric shocks (i.e. without correcting for any cells killed by the transfection procedure).

RESULTS

Effects of trypsin, electroporation buffer and temperature

Cells growing as monolayers must be detached before electroporation. To investigate the effect of exposure to trypsin, CHO cells from a suspension culture were incubated for 10 min in varying concentrations of trypsin before electroporation (using conditions which were optimized by dye staining as described below). No effect on the percentage of permeabilized cells was observed, but there was a striking improvement in the ability of cells exposed to trypsin to recover from the shocks (Table 1) in agreement with other studies [14]. We have observed that massive membrane blebs are associated with cells that are actively resealing (not shown). The effect of trypsin may be due to the removal of cell surface proteins or glycoproteins which impair the ability of cells to reseal. Although non-adherent cells, such as lymphocytes, do not require trypsinization to prepare them for electroporation, exposure to trypsin increases their subsequent survival (our unpublished observations).

It has been suggested that successful permeabilization required the generation of electric fields in cell suspensions at 4 °C [3]. When CHO cells were electroporated under standard electrical conditions at various tempera-

Table 1. Enhanced survival of trypsinized cells

Cells were collected from a suspension culture by centrifugation, washed and incubated with varying concentrations of trypsin for 10 min at 37 °C. The trypsin was stopped by adding complete medium and the samples prepared for electroporation as described in the Materials and methods section. Cells were exposed to nine 8 μs pulses of 3 kV/cm at 5 s intervals, diluted into complete medium and the cells able to be stained immediately measured (permeabilized cells). Final cell viability was determined by the number of cells excluding dye after resealing for 1 h at 37 °C.

Trypsin (μg/ml)	Permeabilized cells (%)	Final cell viability (%)
0	93	56
10	93	59
50	93	80
250	91	85
2500	90	91

tures (0, 4, 10 and 20 °C), no effect was detected: the proportion of initially permeabilized cells was constant (90±2%) as was the percentage of transiently permeabilized cells (76±6%). However we did find that if the cells were incubated at various temperatures in electroporation buffer after exposure to the electric shock, there was a significant difference in the behaviour of the cells held at different temperatures. At 0 °C (Fig. 1*a*) and at 4 °C (not shown), cells did not reseal during incubation in the electroporation buffer, but a continuous decrease in cell viability (measured by dye uptake after the subsequent 1 h incubation at 37 °C in complete medium) occurred. At 20 °C very little resealing occurred during the 60 min in electroporation buffer and at all time points a large percentage of the permeabilized cells were able to recover (Fig. 1*b*). At 37 °C, some cells resealed at early time points in the electroporation buffer, but the membrane repair was incomplete and the cells subsequently became permeable again. At this temperature there was a gradual loss of cell viability (Fig. 1*c*). These results indicated that the maximum period for diffusion of molecules into the electroporated CHO cells was obtained at 20 °C. We have observed, however, that some cell types either reseal to a greater extent than CHO at 20 °C or, in the case of lymphocytic cells, lose viability more rapidly.

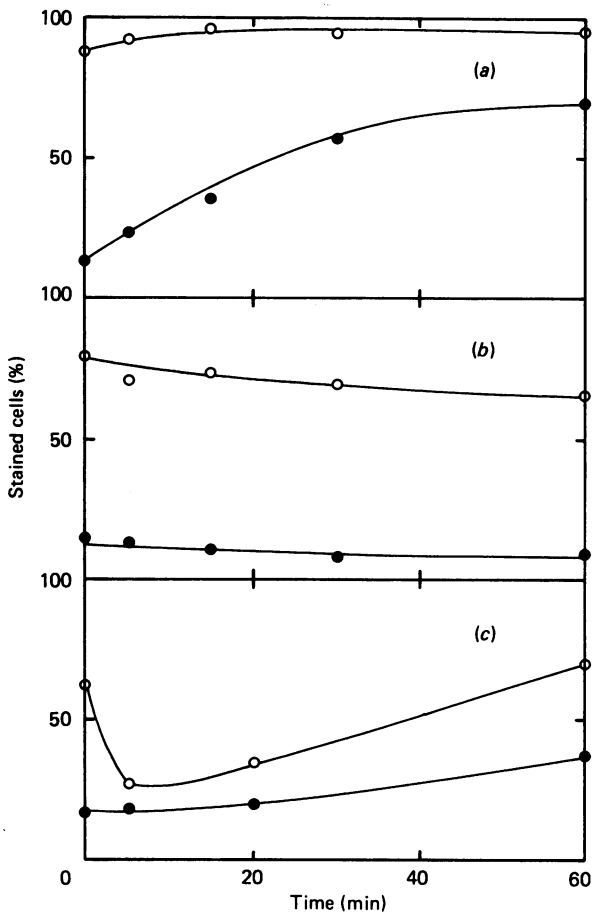

Fig. 1. Viability of cells during incubation at different temperatures after electroporation

Samples of cell suspensions were subjected to nine 8 μs pulses of 3.6 kV/cm at intervals of 5 s at room temperature. Immediately after, they were placed at the stated temperature. At various times, complete medium was added to separate samples and the percentage of stainable cells determined immediately (○) and after a subsequent 1 h incubation at 37 °C (●). Cells at: (*a*) 0 °C, (*b*) 20 °C, and (*c*) 37 °C.

Effect of DNA concentration and conformation

Transformation frequency was determined with varying concentrations of pSV2*neo* under conditions that gave optimal transient permeabilization, determined by dyes. Under these conditions transformation of CHO to a G418-resistance phenotype reached a maximum of nearly 0.4% with pSV2*neo* present at 10 μg/ml (Fig. 2). A gradual decline in frequency of transformation above this concentration of pSV2*neo* may be due in part to reduced cell viability, as higher concentrations of DNA are toxic to permeabilized cells (Fig. 2). However, this may not be the only explanation as it appeared that at least one step in the process of stable transformation is saturable: when increasing concentrations of carrier DNA were added to a fixed concentration of pSV2*neo* (10 μg/ml) the transformation frequency was reduced to a greater extent than was the cell viability (Table 2).

The toxicity of DNA was dose-dependent, with nearly all the cells being killed when electroporated (under the stated conditions) with DNA at concentrations of 200 μg/ml and above (Fig. 3); DNA (up to 500 μg/ml) was not toxic to cells unless they had been electroporated. These results were not due to toxic contaminants of the DNA, as deoxyribonuclease digestion, followed by inactivation of the enzyme by heating at 65 °C for 5 min, abolished the toxicity: digestion products equivalent to 150 μg of DNA/ml gave only 18% non-viable cells, the same as controls without DNA, whereas DNA incubated without enzyme, heat treated and tested at the same concentration resulted in 93% non-viable cells.

The form of plasmid DNA (whether supercoiled or linear) has been reported to have varying effects on transformation frequency in different studies [1–3,7,9]. Although we have not examined this variable in detail, we found little difference in transformation efficiency of

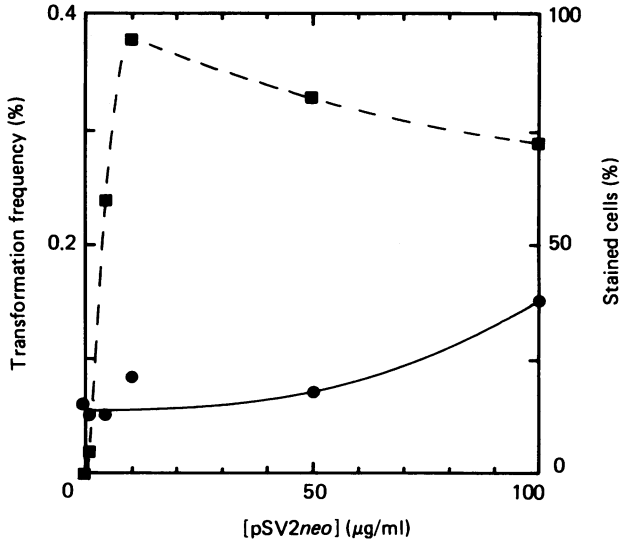

Fig. 2. Transformation frequency after electroporation with varying concentrations of pSV2*neo*

Cells suspended in varying concentrations of pSV2*neo* were subjected to nine 8 μs pulses of 3 kV/cm at intervals of 5 s. Following a 1 h incubation at 20 °C, complete medium was added and aliquots were assayed for transformation frequency (■) and ability to reseal within a subsequent 1 h incubation at 37 °C (●).

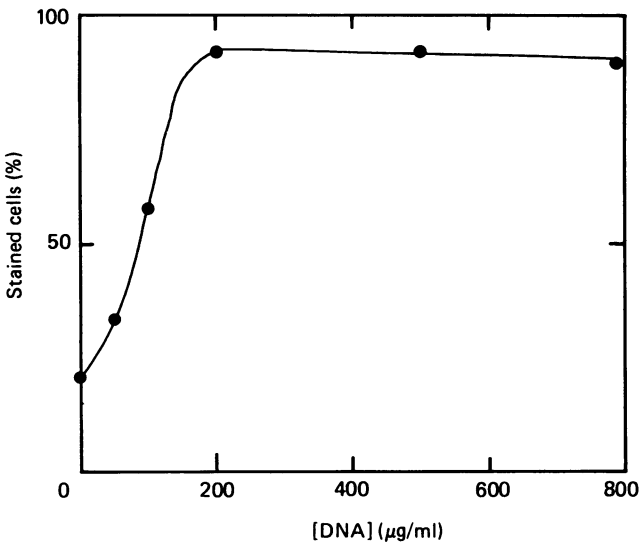

Fig. 3. Toxicity of DNA present during electroporation

Cells suspended in salmon testes DNA at varying concentrations were subjected to nine 8 μs pulses of 3 kV/cm at intervals of 5 s. Under these conditions 90–95% of the cells were permeabilized. The cell suspensions were incubated for 1 h at 20 °C before adding complete medium, incubating for a further 1 h at 37 °C and determining the percentage of non-viable cells by erythrosin B staining.

Table 2. Suppression of transformation by carrier DNA

Cells suspended in 10 μg of pSV2*neo*/ml with varying concentrations of salmon testes DNA were subjected to nine 8 μs pulses of 3 kV/cm separated by intervals of 5 s. The samples were incubated for 1 h at 20 °C before adding complete medium as described in the Materials and methods section. For the sample containing pSV2*neo* alone these electroporation conditions resulted in 94% of the cells being initially permeable and 69% transiently permeable. For each sample the number of cells killed by the procedure (determined by dye uptake after 1 h in complete medium) and the frequency of stable transformation was determined.

Total DNA concentration (μg/ml)	Non-viable cells (%)	Transformed (%)
0	25	0.000
10	56	0.226
20	56	0.136
30	50	0.090
50	50	0.057
100	65	0.055

CHO when electroporation was carried out with 10 μg of pSV2*neo*/ml in the supercoiled (0.28% transformation), relaxed circular (0.26%) or linear (0.25%) forms. The majority of DNA, as determined by agarose gel electrophoresis, was in the stated conformation (not shown).

Effect of field strength on transformation frequency

Increasing the field strength above a threshold value caused a sharp rise in the percentage of cells able to take up dye immediately after the electric shocks (Fig. 4*a*). By 2.5 kV/cm nearly 100% of the cells were permeabilized. In agreement with the previous results, very few cells resealed during the incubation at 20 °C in electroporation buffer in the presence of pSV2*neo*, although some resealing was detectable with field strengths at or below those causing maximum staining (Fig. 4*a*). Loss of viability gradually increased for samples where all the cells had been initially permeabilized, until nearly the whole population was killed at 8 kV/cm (Fig. 4*a*). By subtracting the percentage of killed cells from those stained initially, a value for the percentage of transiently permeabilized cells could be obtained. This reached a maximum of 73% at 2.5 kV/cm and declined thereafter, due to the voltage-induced cell killing (Fig. 4*b*). In other experiments, we have found that very similar staining results were obtained when the cells were not incubated in electroporation buffer before permitting resealing and also when the small amount of pSV2*neo* DNA was omitted (not shown).

Selection for transformants resistant to G418 from aliquots of these same suspensions showed a sharp peak of maximal transformation frequency which correlated well with the profile of transiently permeabilized cells (Fig. 4*b*). In fact, the transformation peak was narrower than the peak of transient permeabilization and was centred at a higher voltage within the peak of transiently permeabilized cells. These results indicate that, after a threshold voltage, transformation frequency increases with field strength only to be limited by the loss of viability of cells at high voltages. We found a similar correlation between transient permeabilization and transformation in other cell lines and, in CHO, with transient expression of the pRSV*cat* gene (our unpublished observations).

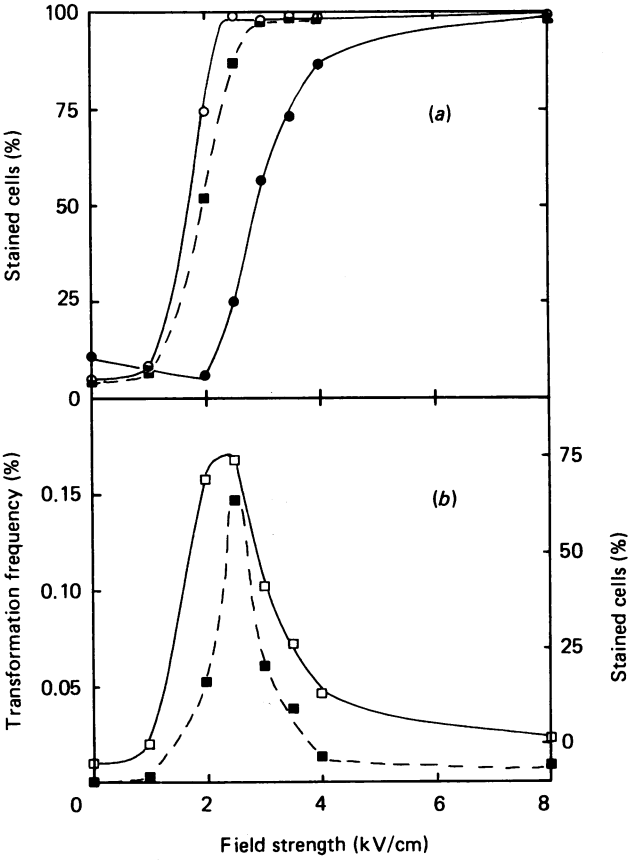

Fig. 4. Effect of field strength on permeability and transformation frequency

Cells suspended in 10 μg of pSV2*neo*/ml were subjected to nine 8 μs pulses of varying amplitude at intervals of 5 s. Before addition of complete medium, the suspensions were incubated for 1 h at 20 °C. Samples electroporated at varying field strengths were assayed for permeability and transformation as described in the Materials and methods section. (*a*) Staining results: immediately after the electric shocks (○), after 1 h at 20 °C (■), and after a subsequent 1 h at 37 °C in complete medium (●). (*b*) Transiently stained cells (□) and transformation frequency (■).

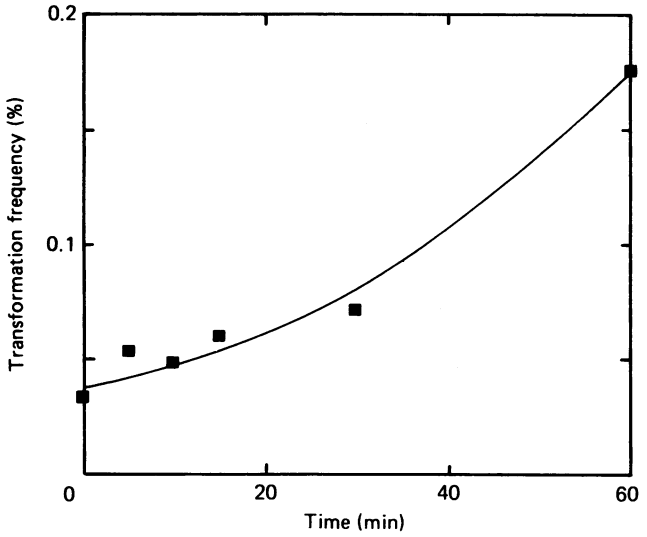

Fig. 5. Effect of incubation for various times in the presence of pSV2*neo* after electroporation

Cells suspended in 10 μg of pSV2*neo*/ml were subjected to nine 8 μs pulses of 3 kV/cm at intervals of 5 s. After various times of incubation at 20 °C samples were removed to assay for transformation frequency (■).

Incubation time in presence of DNA

In experiments described above, permeabilized cells had been incubated for extended periods of time to allow the DNA to diffuse into the cells. However, when cells were placed into complete medium immediately after exposure to electric shocks in the presence of pSV2*neo*, with little time for diffusion of DNA into the cells, a significant number of transformants were obtained (Fig. 5). The number of transformants increased slowly during the subsequent incubation at 20 °C, doubling approximately every 30 min. No time-dependent increase was observed when the permeabilized cells were incubated at 0 °C or 37 °C (our unpublished observations). The lack of time-dependency at these temperatures can be explained by the behaviour of the cells (Figs. 1*a* and 1*c*).

Surprisingly, if the DNA was added immediately after the electric shocks (within 30 s of the last pulse) and incubated with the permeabilized cells for 1 h at 20 °C, very few transformants were obtained. Under these conditions only 0.002% of the cells were transformed, compared with 0.175% for cells electroporated in the presence of pSV2*neo*, despite the fact that the cells were still permeable to dyes at the end of this incubation period. This finding, together with the relatively high transformation frequency (0.033%) obtained when no time was allowed for diffusion after electroporation, suggests that the electric field has a direct effect on DNA transfer across the plasma membrane in addition to its ability to generate pores.

Effect of pulse duration

Single pulses of varying duration at 3 kV/cm were applied to cell suspensions in solutions of pSV2*neo*. To investigate the effect of pulse duration alone, the cells were plated immediately in complete medium without incubating the mixture at 20 °C. A progressive increase in the initially permeabilized cells was obtained reaching a maximum at 500 μs (Fig. 6). For pulses up to 200 μs long, nearly all the permeabilized cells were able to reseal. Beyond this point a progressive loss of viability was observed. The maximum number of transformed cells was found to coincide with the peak of transiently permeabilized cells. However, the number of transformed cells relative to the number of transiently permeabilized cells increased progressively with the duration of the pulse (e.g. compare values at 100 and 200 μs, Fig. 6). These results support the idea that the electric field effects transfer of DNA across the membrane as well as permeabilization of the cells.

The low transformation frequency seen in Fig. 6 is due to the lack of incubation after the electric shock. When electric fields of varying cumulative duration, obtained by varying the number or duration of the pulses, were applied in the presence of pSV2*neo*, and the cells were incubated for 1 h at 20 °C before dilution in complete medium, the frequency of G418-resistant colonies showed a sharp peak at a total duration of 100 μs (Table 3). As expected, the transformation was substantially

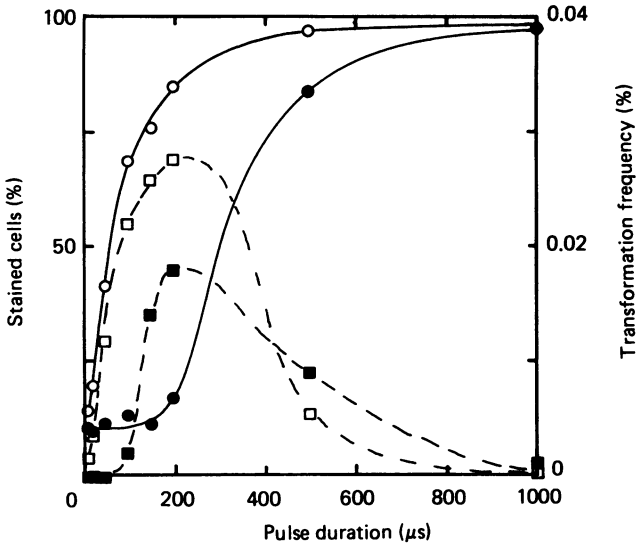

Fig. 6. Effects of a single pulse of varying duration on permeability and transformation frequency

Cells suspended in 10 μg of pSV2*neo*/ml were subjected to a single pulse of varying duration at 3 kV/cm and immediately placed at 37 °C in complete medium. Aliquots were tested for erythrosin B staining immediately (○) and after 1 h at 37 °C (●). An aliquot was also assayed for transformation frequency (■). Transient permeability (□) was calculated as described in the Materials and methods section.

higher than the frequency obtained when no post-shock incubation with DNA was performed (Fig. 6).

There was a rough correlation with transient permeabilization, although the data suggest that for a given degree of permeabilization a smaller number of long pulses may be more effective than the same cumulative duration given as a series of small pulses. In support of this, a single pulse approximately equivalent to the cumulative pulses used for the experiment shown in Fig. 3 resulted in maximum toxicity at 5–10-fold lower concentrations of DNA (our unpublished observations). However, for some cell types, dye staining results showed that it was necessary to apply the required cumulative duration as a series of smaller pulses to allow the capacitors to be recharged between pulses.

Monitoring DNA transfer by its toxicity

Although dyes permit the rapid determination of pore formation by electric shocks, the physicochemical differences between erythrosin B and DNA suggest that transfer conditions for the two molecules will not always coincide. In fact this was shown for the effect of post-shock incubation on transformation (Fig. 5). As high concentrations of DNA were toxic to electroporated cells, it seemed that this property might be used to determine the optimal conditions for DNA transfer in a more direct manner than by using dyes alone.

Samples of CHO were electroporated at various voltages in solution of salmon testes DNA (300 μg/ml) and incubated at 20 °C for 1 h. Complete medium was added and the cells were incubated for a further period of 1 h at 37 °C to allow viable cells to reseal. As a control for cell death due to factors other than intracellular accumulation of DNA, a second set of samples was prepared where the DNA was added within 30 s after exposure to the electric shocks, at the start of the 1 h incubation at 20 °C.

The results show that when cells were electroporated at amplitudes above about 2.5 kV/cm in the presence of DNA, the majority of cells that were initially permeable were killed (Fig. 7). By contrast, cells that were permeabilized before addition of DNA and incubated for 1 h at 20 °C (during which time few of the initially permeabilized cells resealed) were able to survive much higher field strengths; these controls gave results similar to those expected for cells not exposed to DNA at all or only exposed to low concentrations of DNA (see Fig. 4*a*). The difference between the final staining of cells electroporated in the presence of DNA and of cells to which DNA was added afterwards gave a peak of voltage-induced DNA toxicity at 2.5 kV/cm. This value agrees well with the optimum field strength for transformation (Fig. 4). In addition, these results support the idea of electrophoretic transfer of DNA into the cells.

DISCUSSION

The application of intense electric fields to suspensions of cells perturbs the structure of plasma membranes,

Table 3. Effect of number and duration of pulses

Cells suspended in 10 μg of pSV2*neo*/ml were subjected to pulses of varying number or duration at 3 kV/cm with 5 s intervals between pulses where relevant. The samples were incubated for 1 h at 20 °C before adding complete medium. Assays for transformation and permeability were as described in the Materials and methods section.

Cumulative duration (μs)	Number of pulses	Pulse duration (μs)	Frequency of transformation (%)	Transiently permeable cells (%)
72	9	8	0.215	71
90	9	10	0.283	45
100	1	100	0.554	52
180	9	20	0.012	7
200	2	100	0.009	3
400	4	100	0.005	7
450	9	50	0.003	4
900	9	100	0.001	6

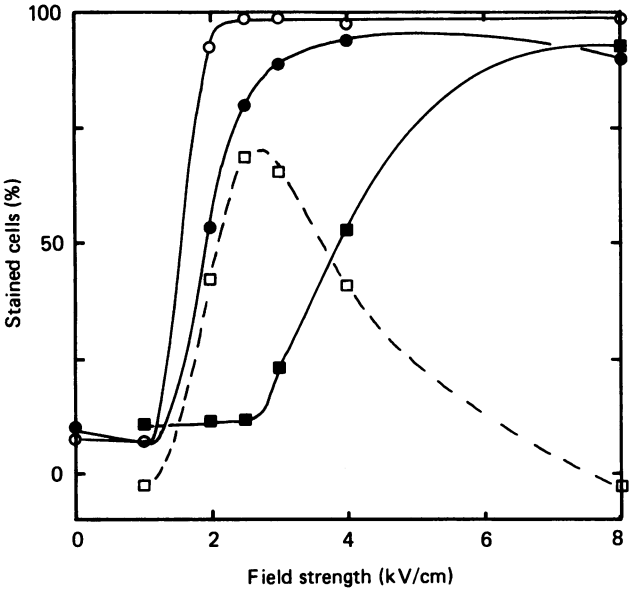

Fig. 7. Variation of DNA toxicity with field strength

Samples were subjected to nine 8 μs pulses of varying amplitude at intervals of 5 s in the presence or absence of 300 μg of salmon testes DNA/ml. DNA was added to the suspensions electroporated in its absence and both sets were incubated for 1 h at 20 °C. Complete medium was added and the samples were incubated for a further 1 h at 37 °C. Assays for permeability were as described in the Materials and methods section. Staining results: immediately after the electric shocks in the presence of DNA (○), after the 1 h incubation at 37 °C in complete medium for cells exposed to electric shocks in (*a*) the presence (●) or (*b*) the absence (■) of DNA, and the difference between (*a*) and (*b*) (□).

resulting in reversible permeability to molecules that would otherwise be excluded. Our results show that the fraction of permeable cells increases with both field strength and pulse duration, in agreement with previous studies on erythrocytes [15–17]. The frequency of stable gene transformation also increased with these two parameters, but was limited by the loss of cell viability under extreme conditions. The voltage-induced cell death was measurable by erythrosin B staining. The failure to detect cell death by Trypan Blue staining [9] is not understood, but may be due to the different dye used. A separate study used Trypan Blue to determine 'viability' by staining immediately after the electric shock [10]; however, that approach ignores the ability of cells to reseal under appropriate conditions. We have successfully monitored transient permeabilization of ten different cell types by the methods described in our report; as expected, the optimum conditions varied with the cell type.

In most cases, we were able to predict the best conditions for stable gene transformation of CHO cells by determining the optimum conditions for transient staining. However, although CHO cells could be maintained completely permeable to dyes for periods of at least 1 h after electroporation, efficient uptake of DNA only occurred if the DNA was present during the application of the electric shocks. Theoretical discussions of the effect of electric fields have only considered the formation of pores in cells [1,14,17] and previous investigations of electric shock-mediated DNA transfer have assumed that the DNA penetrates the permeabilized cells by free diffusion [1,3,7,9,14]; however, efficient diffusion through pores generated by electric fields has been shown to be limited to molecules such as stachyose, well below the size of DNA [16].

The effect of the electric field on the molecules being transferred has not been considered. The electrophoretic mobility of DNA would be considerably greater than that of cells due to the differences in their charge-to-mass ratios. The difference in relative velocities might result in electric field-mediated injection of DNA into cells, in a manner analogous to pulsed-field electrophoresis, with the DNA passing end-on through pores formed by the field. Interestingly, fertilization membranes caused by influx of Ca^{2+} ions only occurred on the anodal side of sea urchin eggs exposed to a single electrical pulse [18]. The opposing electrophoretic migration of the cation and the negatively charged egg would be entirely consistent with our model of electro-injection.

Although we believe that electro-injection initiates the uptake of macromolecules, it is clear from Fig. 5 that the maximum transfer requires a subsequent incubation step, perhaps to permit resolution of complex structures in which the DNA has only partially traversed the plasma membrane. It is also evident that lower-M_r compounds such as neutral sugars and Ca^{2+} [15–18] and erythrosin B (as shown here) are able to transfer in the absence of an electric current through functional pores generated by the electric shock. However, the realization that electrophoretic movement of molecules enhances their transfer across membranes should be useful in the design of experiments to improve transfer of molecules that were inefficiently taken up before.

The necessity to investigate the conditions for electroporation of each cell type was recognized [9]. By measuring the difference in cells stained (after allowing resealing of viable cells) between samples that were electroporated in the presence and absence of toxic concentrations of DNA, it is possible to optimize the various parameters that effect the transfer of DNA across the plasma membrane. In our experience field strength and pulse duration are the critical parameters, the best results being obtained with one or a small number of pulses of the longest duration and highest field strength compatible with viability. Having optimized the electrical conditions in this way, the same test can be used to estimate the appropriate concentration of DNA to minimize toxicity during transformation.

The method of optimizing electric shock-mediated gene transfer described here represents a significant improvement over both stable and transient gene expression assays. As even transient expression assays normally take a minimum of several days to perform, it is the only method available for fine tuning in situations where small fluctuations in cell response occur between cultures harvested on different days, a phenomenon we have occasionally observed.

The procedures we have applied can only monitor the transfer of DNA across the plasma membrane. Other aspects of successful stable transformation such as nuclear localization, integration into the host genome and regulation of gene expression must be considered separately. The observation that up to 70% of the cell population is susceptible to electric shock-mediated

DNA toxicity indicates that dramatic increases in transformation frequencies may be obtainable by investigating the factors regulating intracellular localization and chromosomal integration.

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